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Published in:
Frontiers in Ecology and Evolution

DOI:
10.3389/fevo.2015.00075

Publication date:
2015

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 10 July 2015
doi: 10.3389/fevo.2015.00075

The role of cVA and the Odorant binding protein Lush in social and sexual behavior in *Drosophila melanogaster*

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Specialty section:
This article was submitted to Chemical Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *14 April 2015*
Accepted: *23 June 2015*
Published: *10 July 2015*

Citation:
Billeter J-C and Levine JD (2015) The role of cVA and the Odorant binding protein Lush in social and sexual behavior in Drosophila melanogaster. Front. Ecol. Evol. 3:75. doi: 10.3389/fevo.2015.00075

Social living is beneficial because it allows conspecifics to interact in ways that increase their chances of survival and reproduction. A key mechanism underlying these benefits is the ability to recognize conspecifics; thus, allowing the production of coordinated social interactions. Identification of such individuals is often through chemical communication: the individuals' pheromonal profile indicates their sex, species, and even past experiences. However, we know little about how the chemosensory system of conspecifics detects and how the nervous system processes this information. One of the best documented pheromonal detection mechanisms is that of cis-Vaccenyl Acetate (cVA) made by male *Drosophila melanogaster* and transferred to females during mating. Sensing of cVA by males inhibits courtship behavior toward already mated females. Sensing of cVA on other males also inhibits courtship and increases aggression. In this hybrid review/research article, we discuss the pheromonal system of *Drosophila* putting an emphasis on the molecular and cellular mechanisms involved in cVA sensing by the olfactory system, perception by the nervous system and ultimately the regulation of social interactions. The behavioral effect of cVA is context- as well as experience-dependent leading us to conclude that cVA plays a modulatory role in regulating social interactions rather than being a recognition pheromone. We also provide new behavioral data on the function of the Odorant Binding Protein Lush, which binds cVA in olfactory sensilla and help sensing this chemical. Our data indicate that *lush* may be involved in the sensing of additional pheromones to cVA and suggest the existence of a *lush*-independent cVA detection system. Interpretation of our data in the light of our current knowledge about pheromonal recognition in *Drosophila* indicates that this system is incompletely understood.

Keywords: pheromones, cuticular hydrocarbons, olfactory and gustatory system, sexual behavior, social behavior, aggregation, odorant binding proteins, *Drosophila melanogaster*

Introduction

Recognition of the identity and status of conspecifics permits coordinated behaviors, such as males displaying courtship toward females and not toward males. Several aspects of conspecific recognition in animals are mediated through pheromones: chemical signals produced by one individual that change the behavior of others. The molecular and cellular basis of pheromone-mediated recognition has been extensively dissected in the fruit fly *Drosophila melanogaster* (Billeter and Levine, 2013; Laturney and Billeter, 2014). In this species, pheromones vary in quantity and quality between individuals of different sexes and status. This variation is caused by genetic diversity (Ferveur et al., 1997; Marcillac et al., 2005a; Chertemps et al., 2006, 2007; Fernández et al., 2010), aging (Kuo et al., 2012) and exposure to environmental factors, such as diet (Fedina et al., 2012) and social experience (Butterworth, 1969; Kent et al., 2008; Krupp et al., 2008). The repertoire of pheromones displayed by an individual fruit fly appears to act as a biographical indicator that can be sensed by other flies, thereby informing their social interactions.

Cuticular Hydrocarbons Function in Sex and Species Recognition

Flies produce an excess of 30 different hydrocarbons displayed on the surface of their cuticle hence called cuticular Hydrocarbons (CHs) (Yew et al., 2008; Everaerts et al., 2010; Levine et al., 2010) (**Figure 1A**). These CHs vary qualitatively and quantitatively between males and females as well as between *Drosophila* species (Antony and Jallon, 1982; Jallon and David, 1987). Cells called oenocytes located directly under the cuticle of the abdomen synthesize these CHs in adults (Billeter et al., 2009) (**Figure 1A**). Elimination of the oenocytes results in a dramatic reduction in CHs and a breakdown in recognition (Billeter et al., 2009). Wild-type *D. melanogaster* males display courtship and attempt to mate with both males and females that lack oenocytes (Oe^-) (Billeter et al., 2009); and wild-type males exposed to Oe^- males will court those males rather than display aggression toward them (Wang et al., 2011). Taken together, these data indicate that the Oenocytes produce a signal that conveys sexual identity. Furthermore, males from sibling *Drosophila* species will attempt to court and mate with *D. melanogaster* Oe^- females indicating that Oenocytes also convey species identity (Savarit et al., 1999; Billeter et al., 2009).

Just as removing the Oenocytes from the fly showed us the role of these cells in sexual and species identification, placing specific CHs onto flies can reveal the role of each individual chemical. The recognition of Oe^- flies by social partners can be manipulated by perfuming them with individual CHs. Attraction of *D. melanogaster* males to Oe^- males is blocked by perfuming with the male CH 7-Tricosene (7-T), and restored male aggression (Miyamoto and Amrein, 2008; Billeter et al., 2009; Wang et al., 2011; Thistle et al., 2012; Fan et al., 2013). The attraction of males from other *Drosophila* species to Oe^- females is delayed by perfuming them with the *D. melanogaster* female-specific CH 7,11-Heptacosadiene (7,11-HD). The results of these tests not only revealed the role of CHs in courtship behavior, they also indicated a more general rule for chemical communication in these species. The logic of sexual recognition in *Drosophila* appears to be based on a general attractiveness onto which single sex- and species-specific CHs are superimposed by the Oenocytes to block interactions that reduce evolutionary fitness, such as male–male courtship and interspecies courtship.

Cuticular Hydrocarbons are Detected in Part by the Gustatory System

Although we are starting to understand the chemical signals that indicate an individual's identity, our understanding of how conspecifics sense and perceive these signals is limited. Flies engage in touching via their legs during social interactions, which has been associated with information transfer within groups of flies (Schneider et al., 2012; Ramdya et al., 2015). Part of the information appears to be mechanosensory and related to the sense of touch itself (Schneider et al., 2012; Ramdya et al., 2015). However, touching also has a chemosensory component. For instance, during courtship males tap females with their first pair of legs (**Figure 1A**). The fly legs and body are covered with hairs that contain taste receptors (Vosshall and Stocker, 2007) (**Figure 1E**). The taste receptor Gr32a is located on the first pair of legs (**Figure 1E**). *D. melanogaster* males lacking the gene encoding the Gr32a receptor court females from other species (Fan et al., 2013), court males from their own species (Miyamoto and Amrein, 2008) and have reduced aggression toward conspecific males (Wang et al., 2011; Andrews et al., 2014). *Gr32a*$^-$ mutant males are insensitive to 7-T indicating that Gr32a is a receptor for that pheromone (Wang et al., 2011). Although it probably has a mechanosensory component, one function of tapping during courtship appears to be connected to the chemosensory sensing of CH of conspecific and is in part connected to sex and species recognition (Kohatsu et al., 2011; Fan et al., 2013). The female-specific 7,11-HD pheromone, which is important for species-recognition, seems to be detected by gustatory neurons on the legs. Gustatory neurons expressing the ion-channels *pickpocket-23* and *−25* (*ppk-23* and *−25*) respond physiologically to this pheromone (Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012). However, the role of *ppk* is not yet known: it may act as CH receptors or as effectors downstream of receptors for 7,11-HD and other CHs. Regardless of this complexity, it is clear that the family of Gustatory receptors (Ebbs and Amrein, 2007) as well as a novel family of Ionotropic chemosensory receptors (Koh et al., 2014) are implicated in CH detection in the legs. The observation that receptors for CHs are part of the gustatory and not the olfactory system indicate that CHs are detected upon contact between flies. The use of contact pheromones for recognition makes sense because it allows close association between an identity signal and the actual individual. A pheromone with high volatility would render association with a specific individual difficult, especially under the crowded condition in which flies often find themselves (Spieth, 1974). An important caveat in deducing the volatility of a chemical compound acting as pheromone is that its molecular weight alone is not a good predictor of volatility. There is evidence that CH have some degree of volatility despite their heavier molecular weight compared to most odorants opening the possibility that

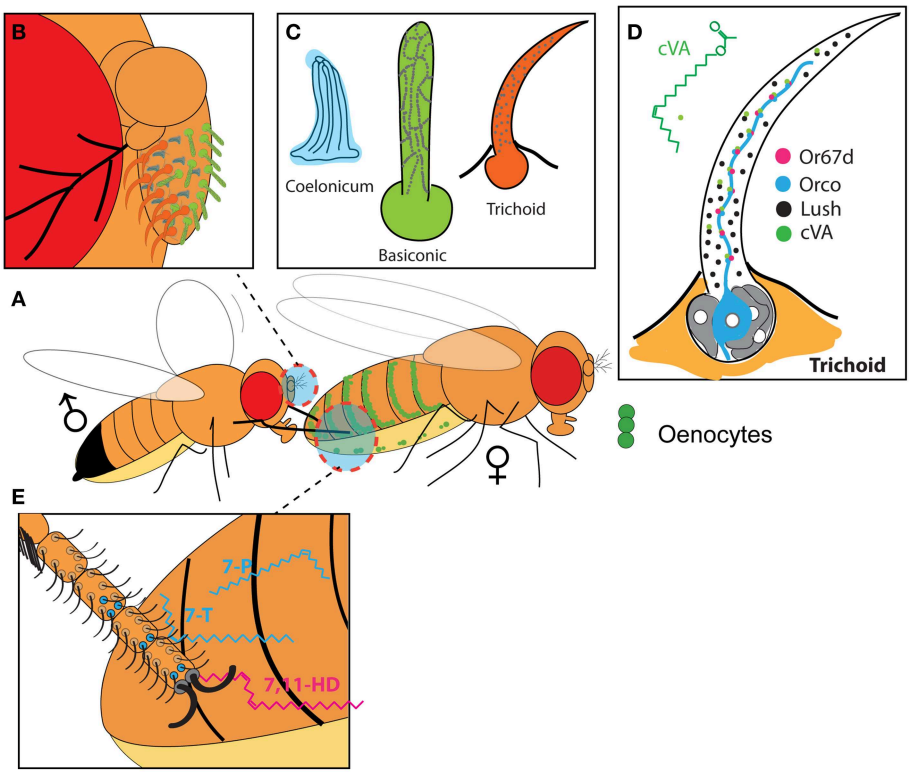

FIGURE 1 | Pheromonal system of *Drosophila melanogaster*. (A) A male engaging in courtship with a female taps her abdomen using his first pair of legs. The female abdomen contains cells called Oenocytes (green dots). **(B)** The olfactory system is located in the third antennal segment, and **(C)** is made of three distinct sensillum-types that differ in morphology. **(D)** The trichoid sensilla are a site of pheromone detection. The pheromone cVA (green dot) enters the sensillum and is bound to the Odorant Binding protein Lush. Lush is secreted by support cells (Gray cells) in the lymph of the sensillum. Lush allows cVA to bind to the Or67d receptor (pink dots) which forms a dimer with the Orco co-recptor (blue dot). Or67d and Orco are both expressed in the dendrite of the olfactory neuron (blue cell) that is housed in the sensillum. **(E)** The male leg possesses sensilla (black hair) that express gustatory receptors, such as Gr32a (blue dots). During tapping, these sensilla are in contact with the surface of the female abdomen, which is coated with cuticular hydrocarbons (blue and pink chemical structures).

they may be also be detected by the olfactory system (Farine et al., 2012).

A Second Pheromonal System: cis-Vaccenyl Acetate

The *D. melanogaster* pheromone whose sensing and perception has been studied in most details is cis-Vaccenyl Acetate (cVA). cVA is produced outside of the oenocytes in a second pheromone-producing organ called the ejaculatory bulb, which is part of the male reproductive tract (Butterworth, 1969; Brieger and Butterworth, 1970). cVA is a unique pheromone in that it has a wide repertoire of behavioral functions. cVA is transferred to females together with the ejaculate during mating (Brieger and Butterworth, 1970). The presence of cVA on a mated female diminishes her attractiveness to males (Jallon, 1984; Kurtovic et al., 2007; Billeter et al., 2009), perhaps a form of chemical mate guarding by the male in an attempt to reduce female promiscuity. However, cVA does not simply function as a male repellant. The inhibitory effect of cVA on male courtship is enhanced by experience; naive males court virgins more than mated females, who have acquired cVA, but males that have the experience of rejection by mated females learn to further suppress courtship specifically toward mated but not virgin females (Siegel and Hall, 1979). This selective suppression is linked to an association by males between the presence of cVA on a female and her rejection behavior (Ejima et al., 2007), which enhances sensitivity to cVA and thus further reduces courtship (Keleman et al., 2012). The response to cVA is thus modified by the experience of prior exposure to that chemical.

The role of cVA extends to the regulation of male–male aggression. cVA increases aggression during sudden encounters between naive males (Wang and Anderson, 2010; Liu et al., 2011), which indicates that it is an aggression stimulating pheromone. However, long-term exposure to cVA reduces male–male aggressiveness (Liu et al., 2011), showing that response to this chemical is modified by experience. cVA thus functions in an experience-dependent manner in both courtship in aggression, whereby the length of exposure to cVA influences the level of these social behaviors.

There is a sex-specific processing component to the response to cVA (Ruta et al., 2010; Kohl et al., 2013). While cVA has a suppressive effect on male courtship, it acts as an aphrodisiac

to females, who mate quicker with males when they sense this pheromone (Kurtovic et al., 2007; Ronderos and Smith, 2010). That cVA enhances male appeal to females but reduces female appeal to males indicates that it has opposite effects on male and female sexual attraction. This suggests that the sexes perceive this pheromone differently. Moreover, females are attracted to lay eggs nearby a source of cVA as the microdistribution of eggs within a food patch correlates with the microdistribution of cVA (Wertheim et al., 2006). Since this behavior can only be produced by females this is one more evidence for the sex-specific processing of cVA. We will see below that the processing of cVA is affected by developmental events that set up different neuronal connections and physiology in males and females (Ruta et al., 2010; Kohl et al., 2013).

Finally, cVA also has a non-sex specific function; in the presence of food, cVA promotes aggregation of both males and females (Bartelt et al., 1985; Wertheim et al., 2002; Xu et al., 2005; Lebreton et al., 2014). Again, prior experience of exposure to cVA has an effect in the context of aggregation. Females who have been chronically exposed to cVA are no longer attracted to aggregate around food laced with cVA (Lebreton et al., 2014). This phenomenon is of interest in the context of social niche construction: the ability to regulate one's social environment. It has been proposed that as cVA concentration increases so too does male–male aggression and that would result in increased dispersion suggesting a model for regulating group size via cVA (Wang and Anderson, 2010). Although the accuracy of this model awaits testing, the discovery of the experience-dependent effect of cVA suggests a modification. Because of the habituation to cVA it is possible that only new comer males would display aggression in a large group size, which might promote dispersal of resident males. As the number of flies who aggregate on a food patch and mate their increases, so does cVA concentration. Males also modulate the amount of cVA they produce or display depending on their social context, offering yet another way to modulate cVA concentration (Kent et al., 2008). Over time males become less aggressive, and mated females might become less attracted to males, both because they became less sensitive to cVA. Other factors are surely also at play such as the availability of food (Simon et al., 2011) and the genotype of males (Saltz and Foley, 2011), but cVA is likely part of a key mechanism in social niche construction.

We conclude that the perception of cVA has both an innate and experience-dependent component affecting social behaviors in *Drosophila* making it difficult to assign a specific function to this chemical.

cVA Has a Combinatorial Role with Food Odorants and Cuticular Hydrocarbons

How can cVA play a simultaneous role in male courtship suppression, female sexual receptivity and egg laying, male aggression and fly aggregation? The answer comes from the observation that cVA has little effect by itself. The first report of this phenomenon came from the work of Bartelt et al. (1985), who found that cVA had to be combined with food in order to attract flies. The aggregative effect of cVA comes from association with yeast-derived odorants (Xu et al., 2005; Schlief and Wilson, 2007; Lebreton et al., 2012, 2014). As yeast-derived odorants such as vinegar are attractive to flies in absence of cVA (Becher et al., 2012) but cVA is not attractive by itself (Bartelt et al., 1985), the role of cVA may be to increase the attractiveness of food rather than render it attractive.

cVA also functions in combination with the CH system. The female pheromone 7,11-HD can decrease the inhibitory effect of cVA on male courtship showing that males evaluate both chemicals when deciding how much to court a female (Billeter et al., 2009; Kohatsu et al., 2011). For male–male aggression, Wang et al. (2011) found that the 7-T pheromone dominantly controls behavioral responses to cVA. 7-T is essential for the aggression-promoting influence of cVA, as a males that cannot sense cVA still displays aggression but a male that cannot sense 7-T does not (Wang et al., 2011). The context-dependent roles of cVA thus depends on a combinatorial effect with other chemicals. In all these contexts cVA increases or decreases an attraction or repulsion that is already there. We conclude that cVA is better described as a gain regulator of the response to other pheromones than a classical pheromone.

cVA is Sensed by the Olfactory System

The third segment of the antenna harbors hair-like structures called sensilla (**Figure 1B**) (Shanbhag et al., 1999), which house the dendrites of one to four Olfactory Receptor Neurons (ORN) (Couto et al., 2005; Fishilevich and Vosshall, 2005). A class of olfactory sensilla called trichoid differ anatomically from the two other main types of olfactory sensilla, basiconic, and coelonic (**Figure 1C**), and are thought to be the general site of volatile pheromones detection (van der Goes van Naters and Carlson, 2007; Vosshall and Stocker, 2007). The only identified pheromone detected by the olfactory system in *Drosophila* is cVA and is indeed detected by a subclass of trichoid sensilla called T1, which only contains one olfactory neuron expressing the odorant receptor Or67d (Couto et al., 2005; Fishilevich and Vosshall, 2005) (**Figure 1D**). In null mutants for the Or67d receptor gene, the electrophysiological response of T1 sensilla to cVA is absent indicating that Or67d is a receptor for that pheromone (Ha and Smith, 2006; Ejima et al., 2007; Kurtovic et al., 2007). The electrophysiological activity of the Or67d receptor relies on a pairing with the Orco co-receptor expressed in all classical olfactory neurons demonstrated by the observation that mutation in *Orco* abrogates the electrophysiological response of T1 sensilla to cVA (**Figure 1D**) (Ha and Smith, 2006; Benton et al., 2007; Jin et al., 2008). cVA is thus sensed by the olfactory system and by a specific type of trichoid sensillum.

How can males and females respond differently to cVA in the context of sex but not in the context of aggregation? Electrophysiological recordings from the T1 sensilla of males and females show the same electrophysiological response suggesting that males and females possess and use the same olfactory channel to detect this chemical (Kurtovic et al., 2007; van der Goes van Naters and Carlson, 2007). Differences in sensing are thus unlikely to be the basis for the sexually dimorphic response to that pheromone. Recordings from interneurons directly downstream of the Or67d Odorant receptor neurons reveals the same electrophysiological pattern of activation between

males and females, indicating no sexual difference in neuronal coding of cVA signal immediately downstream of the first order olfactory neuron (Datta et al., 2008). However, these second order interneurons have different pre-synaptic projections that connect to different third-order neurons in males and females (Datta et al., 2008; Ruta et al., 2010; Kohl et al., 2013). These third order neurons have different developmental origins in males and females and respond differently to cVA (Kohl et al., 2013). The circuitry downstream of cVA sensing is therefore different between males and females, which likely is at the basis of the differences in behavioral responses to this chemical.

Investigation into the neuronal circuitry supporting olfactory information processing has shed light on how cVA can produce differences in behavioral responses between males and females. We mentioned earlier that cVA must be sensed in conjunction with 7-T in order to influence aggression. Mutants for the Gr32a receptor do not show aggression toward male flies, even though they are capable of sensing cVA (Wang et al., 2011). cVA is not sufficient to trigger aggression and this is supported by the observation that male mutants for the Or67d receptor still display aggression toward males (Wang et al., 2011). cVA instead is important for regulating the level of aggression, not the recognition of appropriate opponents. The means through which it does this is beginning to be revealed. Acute exposure to cVA increases aggression via its sensing by the Or67d receptor (Wang and Anderson, 2010; Liu et al., 2011), but long-term or chronic exposure to cVA reduces aggression through activation of a second cVA receptor called Or65a (Liu et al., 2011) expressed in other trichoid sensilla. The way Or65a modulate the response to cVA is elegantly shown by Lebreton et al. (2014). Long-term exposure to cVA activates the Or65a receptor neurons, which inhibits the interneurons that receive input from Or67d via inter-glomerular inhibition (Lebreton et al., 2014). The long-term response to cVA is thus the result of an inhibition of the output neurons of the Or67d receptor by the Or65a olfactory neuron. This system allows gain control over the response to cVA and fine regulation of aggression levels toward individual recognized as males by their expression of 7-T. cVA is thus not a recognition pheromone but a pheromone that modulates the intensity of specific social interactions including aggregation, egg-laying, courtship, and aggression. A key challenge in understanding how cVA is processed will be to understand where the cVA signal(s) is/are integrated in the brain with information coming from other sensory inputs such as food odorants and pheromones as well as touch, vision and internal states and how this integration modulates social behaviors.

Is the Pheromonal System of *D. melanogaster* Fully Understood? Spotlight on the cVA Odorant Binding Protein *Lush*

Despite the wealth of information on the sensing and behavioral function of cVA, it is clear that we lack an understanding of how the sensing of cVA is integrated with that of other pheromones. This ultimately indicates that the logic of pheromonal communication in a species as well studied as *D. melanogaster* is still unclear. For instance, we indicated above that Oe^{-} male and female flies remain attractive to wild-type males despite lacking CHs (Billeter et al., 2009). This suggests the presence of an unidentified pheromone attractive to males that is expressed in both sexes and not produced in the oenocytes. Interestingly, mutants for the Or47b receptor, another Or expressed in trichoid sensilla, are not attracted to Oe^{-} flies (Couto et al., 2005; Fishilevich and Vosshall, 2005; Wang et al., 2011). A newly identified pheromone called methyl laurate made outside the Oenocytes by both males and females is detected by the Or47b and Or88a odorant receptors (Dweck et al., 2015). This new pheromone is detected by a trichoid sensillum like cVA. As Methyl laurate is activatory for male courtship and cVA is mostly inhibitory, these two pheromones might thus have different valence and help modulate male sexual behaviors.

The *lush* mutant was identified based on a behavioral defect in chemosensory attraction to alcohols (Kim et al., 1998) and was later shown to extend to social interactions. Normally, *D. melanogaster* aggregate around a common food source which is enhanced by the emission of cVA by flies already present on the substrate (Wertheim et al., 2006). However, a hypomorphic mutation in *lush* prevents *Drosophila* aggregation to a source of cVA demonstrating a role in social behavior (Xu et al., 2005). Mutations in *lush* results in a dramatically reduced electrophysiological response of the T1 sensilla and Or67d olfactory neuron to cVA (Xu et al., 2005; Ha and Smith, 2006; Gomez-Diaz et al., 2013). Sequence analysis of *lush* reveals that it is an Odorant Binding Protein, a type of protein expressed in chemosensory sensilla thats binds small odorants. Lush is expressed in the lymph of the sensilla, where it binds cVA and might shuttle this pheromone to the Or67d receptor (**Figure 1D**). The necessity of Lush for cVA sensing has led to the proposal that cVA and Lush together form the ligand for Or67d (Laughlin et al., 2008; Ronderos and Smith, 2010). In the context of courtship, a dominant-active mutant of *lush* results in an increased in Or67d neurons electrical spiking in absence of cVA, mimicking to some extend the exposure to cVA (Ronderos and Smith, 2010). Males expressing this mutation dramatically reduce their courtship toward virgin females, indicating that activated Lush can mimic the sensing of cVA on females and inhibit courtship in absence of that pheromone (Ronderos and Smith, 2010). However, this model fails to explain why the Or67d receptor in *Lush* mutants respond to cVA when presented at close range with a 70-fold concentration of cVA (Gomez-Diaz et al., 2013). In absence of Lush, an air stream passing through a source of cVA seven to 70 times more concentrated than that made by a single male (assuming ∼500 ng cVA per male, Lebreton et al., 2014) fails to activate the Or67d receptor (Benton et al., 2007). This model implies a very specific interaction between cVA and Lush, but this fails to explain why Lush is present in all trichoid sensilla, including those that do not respond to cVA (Shanbhag et al., 2001).

The behavioral function of Lush in detecting pheromones has only been studied in the context of a response to cVA (Xu et al., 2005; Ronderos and Smith, 2010), leaving it unclear whether it mediates the detection of other pheromones. *Lush* is not only expressed in trichoid sensilla sensitive to cVA but in all trichoid sensilla (Shanbhag et al., 2001), indicating that this

molecule is involved in the processing of additional odorants, or might have an unappreciated role in all trichoid sensilla. Lush is able to bind *in vitro* to the *Bombyx mori* pheromone Bombykol (Katti et al., 2012). As Bombykol is a long-chain hydrocarbon molecule similar in structure to *D. melanogaster* CHs, this raise the possibility that Lush participates in the detection of these pheromones. The lack of reported courtship defects in $lush^1$ mutants males leaves this possibility untested. Here we investigated the role of *Lush* in sexual recognition and pheromone processing in response to both CH and cVA to investigate its contribution to the sensing of identified *D. melanogaster* pheromones.

Materials and Methods

Fly Strains and Rearing Conditions

All fly strains were reared on medium containing agar (10 g/L), glucose (167 mM), sucrose (44 mM), yeast (35 g/L), cornmeal (15 g/L), wheat germ (10 g/L), soya flour (10 g/L), molasses (30 g/L), propionic acid, and Tegosept in a 12:12 h light/dark cycle (LD 12:12) at 25°C. Virgin adults were collected shortly after eclosion using CO_2 anesthesia and were kept in same-sex groups of 20 in food vials, unless stated otherwise, and aged for 6–7 days. The *Canton-S* strain was used as the wild-type *D. melanogaster* strain. $lush^1$ (Kim et al., 1998) and $orco^2$ (Larsson et al., 2004) mutant flies were placed into the *Canton-S* genetic background. Oenocyte-less (Oe^-) adults were obtained from the progeny of the cross of "+: *PromE(800)-Gal4, tubP-Gal80^{ts}*;+" to "+: *UAS-StingerII, UAS-hid/CyO*;+." Control adults were obtained from the progeny of the cross of "+: *PromE(800)-Gal4, tubP-Gal80^{ts}*;+" to "+: *UAS-StingerII*;+" (Billeter et al., 2009). Progeny from this cross were kept at 18°C until eclosion. Adult progeny were collected at room temperature and kept at 25°C for at least 24 h. Adults were subjected to three heat-treatments at 30°C (on days 2–4) during the day and returned to 25°C overnight. Adults were left to recover from these temperature treatments for 24 h before behavioral experiments.

Cuticular Hydrocarbon Analysis, and Treatment of Flies with Synthetic Hydrocarbons

For CH analysis, flies were anesthetized on ice and placed into individual glass microvials (Varian Inc., Palo Alto, CA) containing 50 µl of hexane spiked with 10 ng/ml each of octadecane (C18) and 10 ng/ml of hexacosane (C26) as internal standards. CHs were analyzed using a Varian CP3800 gas chromatograph with a flame ionization detector as described in Ejima et al. (2007). Varian Star Integrator software (Varian Inc., Palo alto, Ca) was used to quantify compounds based on peak areas.

Application of synthetic compounds was performed as described in Billeter et al. (2009). Control flies were processed as perfumed flies but sham treated by tumbling them in a perfuming vial without Hydrocarbon compounds added. High purity cVA (∼99%) was obtained from Pherobank (Pherobank BV, The Netherlands) and applied at a final dose of 2000 µg/fly.

Courtship and Mating Assays

To determine the mating latency of males with females, one virgin male and female were aspirated into a 35 × 10 mm petri dish layered with food media. Flies where transferred to assay chambers using a mouth pipette, with the tip changed between flies to avoid CH contamination. Experiments began between Zeitgeber time (ZT) 7 and 8 and were housed in a temperature controlled chamber set at 25°C in a 12:12 light:dark cycle. Cameras (Hitachi CCD camera with the Northern eclipse software (v. 7.0) or Canon S10 camera using the ZoomBrowser EX software (Canon) took pictures of the dishes at 2-min intervals from the beginning of the experiment to determine the onset of copulation (Krupp et al., 2008, 2013; Billeter et al., 2012). The mating latency was calculated as the time elapsed between the introduction of the male and female in the dish and the beginning of copulation. A successful copulation was scored when a pair was seen *in copulo* in more than five successive frames (*D. melanogaster* mates for an average of 16 min). Red light was utilized to visualize the chambers during the dark phase.

Courtship assays of male–male *D. melanogaster* were performed in a cylindrical Plexiglas chamber (10 mm diameter by 5 mm depth). Pairs of flies were continuously video-recorded for 30 min from the moment of introduction. One experienced observer scored videos blind to the genotype or perfuming of the flies. A Courtship Index (CI) was determined as the fraction of a 10-min observation period spent by the male exhibiting courtship steps such as following, tapping, wing extension, licking, and attempting copulation. CI started from the first bout of courtship, defined as the first wing extension, and not from the moment of introduction. Flies that did not initiate courtship within 20 min were excluded because their CI could not be accurately determined. The courtship latency is determined as the time between introduction of the male and female to the first sign of courtship. Tester males were collected shortly after eclosion and aged individually for 6–7 day in small vials with diet to avoid reduction in courtship due to male–male courtship habituation.

Statistical Analysis

Statistical analyses were performed with the GraphPad Prism software for Mac (version 5.0, GraphPad software inc.). Mating latency data were log-transformed, and courtship data arcsine-transformed to approximate a normal distribution unless the raw values defined a normal distribution. Normality was tested before and after transformation using Kolmogorov–Smirnov test. *P*-values were determined by ANOVA followed by the *post-hoc* Tukey–Kramer test when comparing multiple groups.

Results

Lush is Required for Males to Sense a Female Stimulatory Compound

To determine the function of *lush* in male reproductive success, we housed a $lush^1$ mutant or *Canton-S* wild-type male with a virgin control female and observed their latency to mate. Mating latency measures how long it takes for a male and a female to begin copulation. As such it measures both the ability of

the male to successfully court and the female's receptivity to his courtship. The mating latency of *lush*^{1} males with control females is dramatically increased compared to *Canton-S* males (**Figures 2A,B**), suggesting that *lush* males are not attractive to females who otherwise mate normally with wild-type males. As *lush* is expressed in the olfactory system, it is likely that *lush*^{1} males fail to sense a female stimulatory pheromone and thus are slow at initiating or maintaining courtship resulting in delayed mating acceptance by females. Alternative hypotheses will be explored in the discussion.

To test the possibility that the female stimulatory pheromone that *lush*^{1} mutant mails fail to sense is a CH, we exposed *lush*^{1} males to Oe$^-$ females. If that pheromone was a CH we would expect *lush*^{1} males to have the same delayed mating latency as with Control females (**Figures 2A,B**). However, exposure of *lush*^{1} males to Oe$^-$ females resulted in a dramatic decrease in mating latency from 2 h with a control female to 10 min with an Oe$^-$ female (**Figures 2A,B**). The interaction between *lush*^{1} males and Oe$^-$ females suggests that Oe$^-$ females provide an attractive signal that is not a CH and that does not required *lush* to be sensed. These results therefore suggest the existence of two attractive female pheromones, one that may be a CH and one that is independent from that system.

We manipulated two factors in these experiments; the presence of functional *lush* in males and the presence of CH in females (**Figure 2**). Although both factors affect mating latency, statistical analysis indicates a significant interaction between the function of *lush* in males and the presence of CH pheromones in females [Two-Way ANOVA: *lush* in males × female CH: $F_{(1, 59)} = 18.97$; $P < 0.0001$; *lush* in males: $F_{(1, 59)} = 136.02$; $P < 0.0001$; female CH: $F_{(1, 59)} = 63.06$; $P < 0.0001$]. This significant statistical interaction indicates that *lush* and the sensing by males of female CH are connected but that this connection is different depending on the presence or absence of both factors. Beside suggesting the existence of two independent female attractive signals, these experiments imply the existence of an inhibitory female CH which does not require *lush* for its sensing. This inhibitory CH pheromone would explain why *lush*^{1} males have such a long mating latency with control females that is dramatically reduced with Oe$^-$ females. These data suggest that Lush may help sensing distinct classes of pheromones in addition to cVA.

Lush is Not Necessary for the Inhibitory Effect of cVA on Male Sexual Behavior

To directly test the function of *lush* in allowing the sensing of cVA, we tested the mating latency of wild-type and *lush*^{1} males with virgin females with a normal CH profile perfumed or not with 2 µg of cVA. This quantity is four times that found on recently mated females (Lebreton et al., 2014) and 30-fold less than the dose of cVA that demonstrably activate Or67d receptor in absence of Lush (Gomez-Diaz et al., 2013). This dose has been previously shown to reduce male mating latency (Zawistowski and Richmond, 1986; Billeter et al., 2009). We tested two factors; the genotype of the males (wild-type or *lush*^{1}) and the presence or absence of cVA on virgin females (**Figure 3**). Wild-type males had a slower mating latency with virgin females perfumed with cVA than with virgin females not perfumed with cVA, consistent with an anti-aphrodisiac role of cVA (**Figure 3A**). *lush*^{1} males were equally slow to mate with cVA or non-cVA perfumed females, confirming that *lush* is required for cVA sensing and that the amount of cVA used to perfume flies is unlikely to artificially activate Or67d in absence of *lush* (**Figure 3A**). Statistical analysis shows a significant interaction between genotype of the males and the presence of cVA on females [Two-Way ANOVA male genotype × cVA on females $F_{(2, 61)} = 20.21$; $P < 0.0001$; *lush*^{1} in males $F_{(1, 61)} = 87.67$; $P < 0.0001$; cVA on females $F_{(1, 61)} = 28.41$; $P < 0.0001$]. The significant statistical interaction between *lush*^{1} mutant males

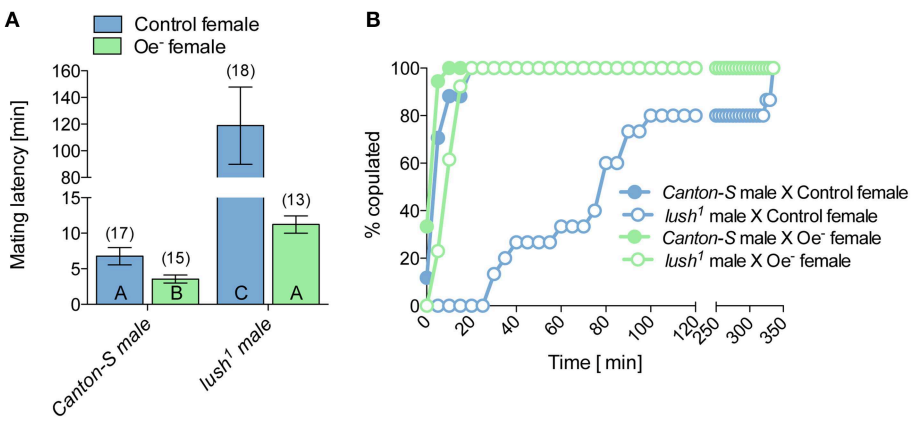

FIGURE 2 | Lush detects a female stimulatory compound. (A) Mean mating latency in pairs consisting of 1 virgin male and female of the indicated genotype. The number of replicates is indicate above the bar graphs. Error bars indicate Standard Error of the Mean (SEM). Bar graphs labeled with same letters are not significantly different from each other as determined by a One-Way ANOVA [ANOVA $F_{(3, 59)} = 74.4$; $P < 0.0001$] followed by Tukey *post-hoc* test. **(B)** Cumulative percentage of pairs of virgin male and female of the indicated genotypes. Data is same as in **(A)** but plotted as cumulative mating to illustrate when most copulations take place.

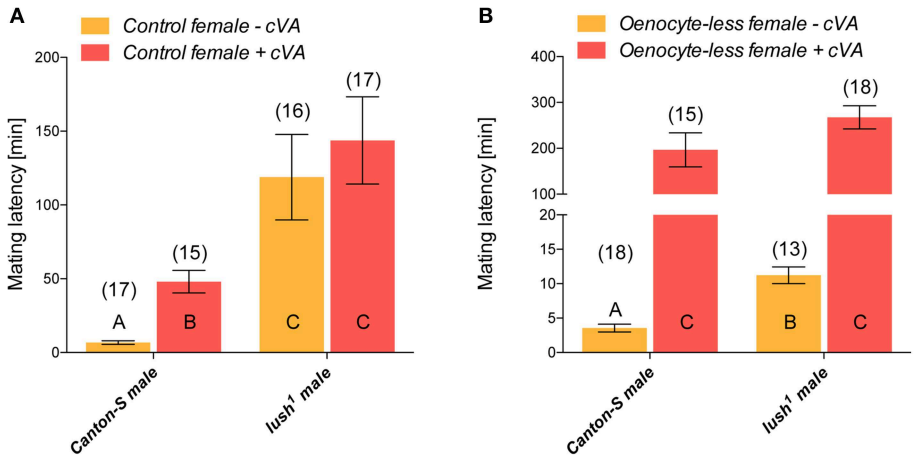

FIGURE 3 | *Lush detects cVA and an unknown inhibitory signal.* **(A)** Mean mating latency in pairs consisting of 1 virgin male of the indicated genotype with one control female perfumed with 2000 ng of cVA or not perfumed. The number of replicates is indicated above the bar graphs. Error bars indicate Standard Error of the Mean (SEM). Bar graphs labeled with same letters are not significantly different from each other as determined by a One-Way ANOVA [ANOVA $F_{(3, 61)} = 46.88$; $P < 0.0001$] followed by Tukey *post-hoc* test. **(B)** Mean mating latency in pairs consisting of 1 virgin male of the indicated genotype with one Oenocyte-less (Oe^{-}) female perfumed or not with 2000 ng of cVA. The number of replicates is indicated above the bar graphs. Error bars indicate Standard Error of the Mean (SEM). Bar graphs labeled with same letters are not significantly different from each other as determined by a One-Way ANOVA [ANOVA $F_{(3, 60)} = 135.7$; $P < 0.0001$] followed by Tukey *post-hoc* test.

and CVA perfuming of females stems from the fact that *lush*^{1} males are slower than wild-type males at mating with both control females that are or not perfumed with cVA (**Figure 3A**). This again suggests that *lush* mediates not only the sensing of CVA but also that of an attractive signal that stimulates mating.

We next tested the mating latency of wild-type males and *lush*^{1} males with virgin Oe^{-} females perfumed or not with cVA (**Figure 3B**). As we previously documented, perfuming Oe^{-} females with cVA dramatically reduced their attractiveness for a wild-type *Canton-S* male compared to perfuming a control female (**Figure 3B**) (Billeter et al., 2009). This suggests that female CHs normally mitigate the anti-aphrodisiac effect of that pheromone (**Figure 3B**). As the *lush*^{1} mutation blocks cVA-sensing (Xu et al., 2005), these mutant males should find both Oe^{-} females that are or are not perfumed with cVA equally attractive and thus mate with them much faster than wild-type males. Strikingly, perfuming Oe^{-} females with cVA also dramatically increased the mating latency of *lush*^{1} males. This result indicates that *lush*^{1} males are still able to detect cVA (**Figure 3B**). The perception of cVA is thus modified by the presence or absence of female CH indicating the presence of a *lush*-independent cVA sensing system. Statistical analysis backs up this conclusion as indicated by a lack of significant interaction between genotype of the male and the presence of cVA on Oe^{-} females, but significant effect of male genotype and cVA on females [Two-Way ANOVA: Interaction male genotype × cVA on females: $F_{(1, 60)} = 3.37$; $P = 0.07$; male genotype: $F_{(1, 60)} = 24.14$; $P < 0.0001$; cVA on females: $F_{(1, 60)} = 347.77$; $P < 0.0001$].

Lush is Required for the Sensing of Non-oenocyte Derived Stimulatory Factors

Our data on *lush*^{1} male mating latency with females indicate that *lush* is partly required for the response to the anti-aphrodisiac cVA but also to attractant pheromones: one that is made by the Oenocytes and thus likely to be a CH and one that belongs to an unidentified pheromonal system. To investigate the relationship between *lush* and the sensing of courtship stimulating compounds, we made use of the fact that Oe^{-} males trigger strong courtship by wild-type *Canton-S* males (Billeter et al., 2009). Oe^{-} males are devoid of CH but still express cVA at wild-type levels (Billeter et al., 2009) showing that cVA is not sufficient to suppress male courtship (**Figure 4A**).

We first tested the courtship behavior of wild-type, *lush*^{1} males or *orco*$^{-}$ males who lack a functional classical odorant receptor repertoire (Larsson et al., 2004; Benton et al., 2006), toward control and Oe^{-} males. Surprisingly, we observed no difference in CI between male genotypes: none of these males courted control males and all of them courted Oe^{-} males vigorously [Two-Way ANOVA: Tester male genotype: $F_{(2, 134)} = 0.09$; $P = 0.9$; Target male CH: $F_{(1, 134)} = 144.47$; $P < 0.0001$] (**Figure 4A**). This indicates that *lush* and *Orco* are not necessary to sense inhibitory pheromones made by the Oenocytes because they court control males at wild-type levels. Although they courted Oe^{-} males at similar intensities than *Orco*$^{-}$ and wild-type males (**Figure 4A**), the time to first bout of courtship (the courtship latency) of *lush*^{1} males toward Oe^{-} males was delayed compared to that of wild-type and *orco*$^{-}$ males (**Figure 4B**). The delayed courtship latency of *lush*^{1} mutants indicates that *lush* has a role in the sensing or perception of an attractive pheromone that is not made in the Oenocytes that stimulates courtship initiation.

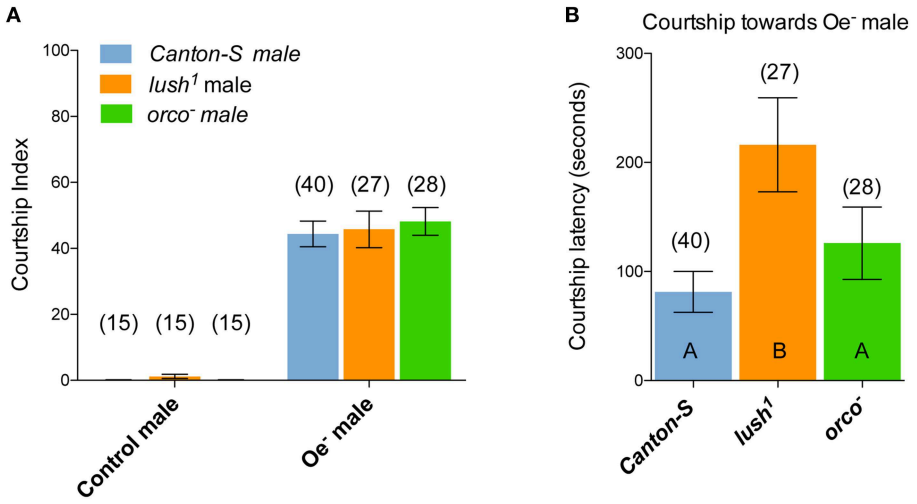

FIGURE 4 | *Lush* is not required for the sensing of identified stimulatory male or females CHs. (A) Male–male courtship index in pairs consisting of 2 virgin males of the indicated genotypes. **(B)** Latency to initiate courtship in males of the indicated genotypes with Oe^{-} males. The number of replicates is indicated above the bar graphs. Error bars indicate Standard Error of the Mean (SEM).

Discussion

Our experiments provide behavioral evidence that the Odorant Binding Protein *lush* is involved in pheromonal detection beyond that of cVA. Further, it shows that *lush* is not always necessary for the behavioral effect of cVA suggesting the existence of a *lush*-independent cVA sensing system. These data begin to explain the expression pattern of *lush* in trichoid sensilla that are not sensitive to cVA and thus suggest that *lush* participates in sensing more pheromones than just cVA. Additionally, our experiments suggest the existence of a complex pheromonal recognition system in *D. melanogaster* and of unidentified pheromones.

Lush is Required for Sensing a Courtship Stimulating Factor

Virgin females lack cVA yet *lush*^{1} males have abnormally long mating latency with those females (**Figures 2A,B**). In absence of courtship data, delayed mating latency could be alternatively interpreted as the female not being willing to mate despite the best effort of *lush*^{1} males or that *lush*^{1} males do not exhibit strong courtship toward those females delaying mating acceptance. Direct observation of the courtship behavior of *lush*^{1} males toward Oe^{-} males shows that *lush*^{1} males can produce level of courtship similar to wild-type males, but that they are delayed in initiating courtship (**Figures 4A,B**). We thus favor the hypothesis that *lush*^{1} males have delayed mating latency with females because they are slow at initiating courtship. This would indicate that *lush* is required to sense a stimulatory factor from wild-type virgin females. Because *lush* is expressed in the pheromone-sensing part of the olfactory system (Kim and Smith, 2001; Shanbhag et al., 2001; Benton et al., 2007), this factor is likely to be a volatile and a pheromone. If there was only one female stimulatory pheromone and it was a CH, *lush*^{1} males should be equally slow at mating with control females than Oe^{-}. They are however much faster indicating the presence of a second non-CH stimulatory pheromone that does not require *lush* to be sensed. The proposal that females possess two attractive pheromonal systems fails to explain the dramatic increase in mating latency of *lush*^{1} with Control but not with Oe^{-} females. This behavioral difference reveals the existence of an inhibitory pheromone made by the oenocytes. This conclusion is in line with our previous report that wild-type males mate quicker with Oe^{-} females than control males indicating that the female oenocytes produce a pheromone with an inhibitory effect on males mating latency (Billeter et al., 2009). The extremely delayed mating latency of *lush*^{1} males with control females would thus be caused by two factors: an inability to sense a non-oenocyte-derived stimulatory pheromones, amplified by the ability to sense an inhibitory oenocyte-derived pheromone. We propose a model in which a balance between one repressive and two stimulatory female pheromones allows to fine tune male–female recognition by making attractiveness a graded response (**Figure 5A**).

This model however does not take into account the possibility that males regulate their behavior based on sensing their own cVA levels. Sensing by the male of his own cVA may normally interact with female pheromones determining his own level of sexual arousal. While it is an untested hypothesis, testing it would be difficult for two reason. The first is that sensing of cVA by males normally reduces their courtship and aggression levels (Kurtovic et al., 2007; Wang and Anderson, 2010; Liu et al., 2011). A mutation affecting a male's ability to sense his own cVA could thus be expected to result in an increased courtship toward both males and females, but that is not the case in our experiments (**Figures 2**, **4**). Second, it is unlikely that males can continuously sense their own cVA because it only seems to be released in the environment during social interactions indicating that males can control the release of this compound (Everaerts et al., 2010).

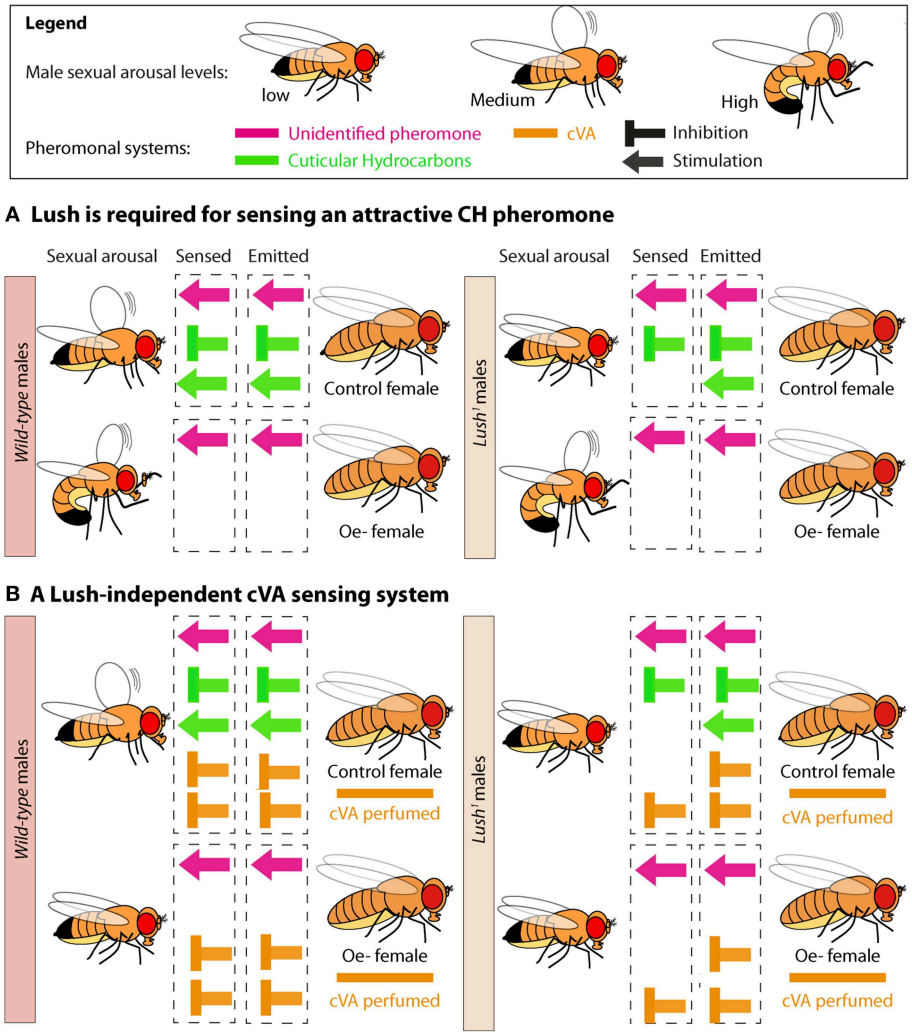

FIGURE 5 | Model of female pheromones sensing by males during courtship. (A) A balance between one repressive and two stimulatory female pheromones fine tunes male–female recognition and makes attractiveness a graded response. *lush* mutant males fail to perceive one stimulatory and one inhibitory cuticular hydrocarbon pheromone made by the Oenocyte (Oe), but still sense the second stimulatory pheromone not originating from the oenocytes. **(B)** *lush* mutant males may suppress courtship toward females perfumed with cVA because they can still sense cVA via a second sensory channel. The strong inhibitory effect of cVA-perfuming on Oenocyte-less (Oe^{-}) females would be linked to a failure by males to sense the stimulatory pheromone coming from the CH system.

A *Lush*-independent cVA Sensing System

The role of *lush* in cVA sensing is well documented. However, the suppression of $lush^1$ male mating latency with cVA-perfumed Oe^{-} females shows that these males can still sense cVA. This observation leads us to suggest the existence of a *lush*-independent cVA sensing system (**Figure 5B**). As *lush* is expressed in all trichoid sensilla (Shanbhag et al., 2001), it is unlikely that this secondary cVA system is the Or65a receptor, which has been shown to respond to chronic exposure to cVA and dampen response by the Or67d receptor (Liu et al., 2011; Lebreton et al., 2014). Despite a lack of electrophysiological data, it is possible that *lush* would impact the function of Or65a, given that it is expressed in the trichoid sensilla that house this receptor (Shanbhag et al., 2001; Couto et al., 2005). So what could this secondary system be? Thistle et al. showed that cVA can activate *ppk23*-positive taste neurons on the male foreleg (Thistle et al., 2012). *lush* mutant males may suppress courtship toward Oe^{-} females perfumed with cVA because they can still sense cVA through their taste system. The reason why $lush^1$ males are strongly inhibited by cVA perfumed control females would be linked to a failure to sense the stimulatory pheromone coming from the CH system (**Figure 5B**).

An alternative explanation for our findings is that the dose of cVA we used can activate Or67d in absence of Lush. It has been shown that a 30-fold dose applied on a piece of paper and brought a close proximity to the T1 sensilla can activate electrophysiological response of the Or67d receptor in absence of Lush (Gomez-Diaz et al., 2013). While we perfumed flies

with a much lower dose (four-fold higher than natural levels) we really do not know how much cVA is normally available to males during courtship given that most cVA in mated females might be present inside the female and not outside (Lebreton et al., 2014). We therefore do not know in how much excess our cVA perfuming, which is applied on the surface of the female, is compared to natural levels. This illustrates how little we really know about the location and availability of cVA in general.

Pheromone Processing in *Drosophila*

The interaction of *lush* mutant males with female pheromones reveals an unexpected complexity in pheromone processing in *Drosophila*. Compounds like cVA are detected by two separate receptors in the olfactory system and may also be sensed by the taste system (Ha and Smith, 2006; Kurtovic et al., 2007; van der Goes van Naters and Carlson, 2007; Liu et al., 2011; Thistle et al., 2012; Lebreton et al., 2014). Detection of the same pheromone by both the olfactory and taste systems may increase the range of functions of a single compound. For instance, at longer range the smell of cVA combined with the smell of food triggers attraction to food substrate already occupied by flies (Bartelt et al., 1985; Wertheim et al., 2002; Lebreton et al., 2012). In the meantime, the taste system would not be able to detect low concentrations of cVA at such a long distance. At close contact, male or female CH and cVA might be sensed by both the gustatory and olfactory systems (**Figure 1A**). Pheromonal detection by the olfactory systems might indicate the environmental concentration of cVA allowing to determine the number of individual in a group and the taste system being more directed would identify specific social partners. Integration of these two senses would result in modulation of the intensity of social interactions with a specific individual, while taking into account the social environment. This is perhaps not so far fetched as pheromones like 7-T and cVA have a degree of volatility and can even be deposited on the substrate acting as good environmental indicator of group composition and size (Farine et al., 2012). There are also evidences that females pheromones are perceived both by the olfactory and taste system (Inoshita et al., 2011).

Another principle of pheromonal recognition is that the stimulatory pheromones of females are matched by inhibitory pheromones (**Figure 5A**). This system would allow for a graded response by males to female pheromones. This would explain why the main stimulatory CH of females 7,11-HD is expressed at different levels in different strains of *D. melanogaster* and elicits different level of courtship response by males from different strains (Marcillac et al., 2005b; Grillet et al., 2012; Pischedda et al., 2014). This difference in level does not make sense in terms of the function of 7,11-HD as a species-specific pheromone inhibiting courtship from males from different species because amounts of 7,11-HD as low as 70 ng completely block mating with males of sibling species (Marcillac and Ferveur, 2004; Billeter et al., 2009). Yet Strains of *D. melanogaster* have concentration of 7,11-HD far exceeding this dose [e.g., Canton-S: ca. 370 ng 7,11-HD (Billeter et al., 2009)]. Females of many strains produce amounts of dienes that largely exceed the threshold required for mate preference. A view in which pheromones counterbalance each other would help resolve this issue. Greater amount of 7,11-HD might for instance reduce the inhibitory effect of cVA (Billeter et al., 2009) in populations where males synthesize large amounts of cVA. These differences might also come from individual variation in the sensitivity of the male chemosensory system to each of these pheromones (Pischedda et al., 2014).

The pheromonal system of *Drosophila melanogaster* is an excellent system in which to study the basic mechanisms underlying the regulation of social interactions. As discussed in the introduction of this article, investigation of this system has allowed identification of several chemicals acting as pheromones, the cells and enzymatic pathways that produce these pheromones, the sensory neurons that detect them, and has even offered a glimpse at the neuronal circuitry that act downstream of the sensing of these chemicals. It is becoming clear that social interactions are not normally regulated by single pheromones, but by the integration of several signals. The challenge now is to understand where and how the sensing of different pheromones, environmental chemicals and other sensory cues such as vision (Agrawal et al., 2014) are integrated in the brain and how this integration regulates social interactions. This challenge is complicated by the fact that not all *Drosophila* pheromones have been identified as indicated by behavioral data and the existence of several orphan chemosensory receptors that seem to be sensitive to unidentified pheromone (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012; Koh et al., 2014). New behavioral assays and new analytical chemical methods are revealing the existence of additional pheromone classes, such as larval (Farine et al., 2014; Mast et al., 2014) and male (Yew et al., 2009) pheromones, and very recently an attractive pheromone found in both males and females and several *Drosophila* species (Dweck et al., 2015) that is likely to be the long-anticipated non-sex and non-species-specific attractive compound made by *Drosophila* species (Savarit et al., 1999; Billeter et al., 2009) making this field exciting, fast evolving and far from resolved.

Acknowledgments

We thank Bloomington *Drosophila* Stock Center for fly stocks. We thank Meghan Laturney and two referees for feedback on the manuscript. This work was supported in parts by grants from the Canadian Institute for Health Research, the National Science and Engineering Research Canada, Canadian Institute For Advanced Research and the Canada Research Chair program to JL and the Swiss National Fund and Dutch organization for scientific research (NWO) to JB.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.